bio-protocol Preprint

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Single nuclear capture, sequencing, and data analysis

MN Minh Q Nguyen NR Nicholas Ryba SD Steve Davidson

Updated date: Jul 11, 2022

An abbreviated version of this protocol was published in eLIFE in Nov 2021

Single-nucleus transcriptomic analysis of human dorsal root ganglion neurons

DOI: 10.7554/eLife.71752

Detailed protocol

Isolation of neuronal nuclei from human DRGs (or tough to dissociate tissues):

- Freeze tissues on dry ice and store at -80^{O}C until use. Alternatively, cut tissues into small pieces (1-2 mm) and incubate in RNAlater (ThermoFisher, cat# AM7021) overnight at RT
- Remove excess RNAlater and freeze tissue pieces in a microfuge tube on dry ice. Store at -80^{O}C until use
- On day of 10x run: prepare homogenization buffer (250 mM sucrose, 25 mM KCl, 5 mM MgCl2, 10 mM Tris, pH 8.0, 1 µM DTT, 0.1% Triton X-100 (v/v). Cool on ice. Stock solutions can be prepared ahead of time.
- Cool down all necessary equipment and solutions. Cool Spectrum™ Bessman Tissue Pulverizer (ThermoFisher, cat#08-418-3) in liquid nitrogen or dry ice
- Add frozen tissues to Spectrum™ Bessman Tissue Pulverizer (ThermoFisher, cat#08-418-3) in liquid nitrogen or dry ice
- Pulverize tissues and transfer to glass dounce homogenizer (Fisher Scientific, cat#357538) containing 1ml of cold homogenization buffer
- Homogenize tissues in glass dounce homogenizer in 1ml of cold homogenization buffer: 5 strokes with “loose” pestle and ~10-20 strokes with “tight” pestle … on ice
- Filter through 40 µm cell strainer (ThermoFisher Scientific, cat# 08-771-1)
- Transfer to microfuge tube (low bind; Sorenson BioScience, cat# 11700) and spin @ ~300-800g at 4^{O} for 5-8 mins (determine what speed and time are best for your tissues)
- Remove supernatant and resuspend pellet in 500 µl of PBS + 1% BSA + SUPERaseIn RNase Inhibitor (0.2 U/µ; ThermoFisher Scientific, Cat# AM2696)
- Incubate on ice for 10-15 mins
- Add rabbit polyclonal anti-NeuN antibody (Millipore, cat#ABN78) 1:4000 to 1:5000
- Incubate with rotation at 4^{O} for 30 mins
- Spin @ 300-800g at 4^{O} for 5-8 mins
- Remove supernatant and “wash” by adding 1 ml PBS + 1% BSA + SUPERaseIN RNase inhibitor
- Spin @ ~300-800g at 4^{O} for 5-8 mins
- Resuspend pellet in 80 µl of PBS + 0.5% BSA + 2mM EDTA. Volume is for 10^{7} total cells. Adjust volumes accordingly if you have more total cells.
- Add 20 µl of anti-rabbit IgG Microbeads (Miltenyi Biotech, cat# 130-048-602). Volume is for 10^{7} total cells. Adjust volume of microbeads accordingly if you have more total cells.
- Incubate at 4^{O} 15-20 mins
- “wash” by adding 1 ml of PBS + 0.5% BSA + 2mM EDTA
- Spin @ ~300-800g at 4^{O} for 5-8 mins
- Remove supernatant and resuspend in 0.5-1 ml PBS + 0.5% BSA + 2mM EDTA
- Load onto LS column (Miltenyi Biotec, cat# 130-042-401) – follow instruction from manufacturer:
  - put column on magnetic MidiMACS separator (Miltenyi Biotec, cat#130-042-302; MACS MultiStand (cat#130-042-303), add ice cold 3 ml of buffer (PBS + 0.5% BSA + 2mM EDTA) to equilibrate
  - Add nuclei to column
  - Wash column with 3 ml ice cold buffer (3x)
  - Remove column from magnet and elute in 5 ml of same buffer using plunger that comes with the LS column. Elute on ice into 15 ml Falcon tube.

- Spin @ 500g at 4^{O} for 10 mins
- Remove supernatant
- Resuspend in 1.5 ml of PBS + 1% BSA + SUPERaseIn RNase Inhibitor.
- Spin @ 300-800g at 4^{O} for 5-8 mins
- Resuspend in 1-1.5 ml of PBS + 1% BSA + SUPERaseIn RNase Inhibitor
- Strain through 35 µm cell strainer (Falcon 352235) to remove any nuclei clumps.

Alternatively, use Ultra-Turrax (Laboratory Supply Network, Inc., cat#IKA:3737001) on setting 1 for 10-45 sec on ice (adjust time accordingly … depending on how many nuclei you have)

- 1st count: Stain 5-10 µl with trypan blue and count (using a hemocytometer) also check for clumps

- Transfer to low bind microfuge tube and spin @ ~300-800g at 4o for 5-8 mins
- Remove supernatant and resuspend in desire volume with PBS + 1% BSA + SUPERaseIn RNase Inhibitor … volume based on the first count.
- *Usually I try to go for 1000 nuclei/µl (if there are enough nuclei). I also assume that the first count is not that accurate (too diluted), so usually resuspend nuclei in ½ the calculated volume.*
- Stain 5-10µl with trypan blue and do a 2nd count. Adjust volume to desire nuclei concentration.
- Optional: do a 3rd count. This should be close to the 2nd count.
- Load appropriate number of nuclei onto 10X chip.

Homogenization Buffer:
250 mM sucrose, 25 mM KCl, 5 mM MgCl2, 10 mM Tris, pH 8.0, 1 µM DTT, 0.1% Triton X-100

** *Can also use EZ PREP buffer (Sigma, Cat #NUC-101) as the homogenization buffer.*

This is a modification of the protocol described in Nguyen et al (2019) Stereotyped transcriptomic transformation of somatosensory neurons in response to injury eLife 8:e49679

Data analysis
10x chromium data were mapped using CellRanger to a pre-mRNA modified human genome (GRCh38.v25.premRNA). Data analysis used the Seurat V3 packages developed by the Satija lab and followed standard procedures for co-clustering (Stuart et al., 2019). For sn-RNA sequencing experiments cell filtering was performed as follows: outliers were identified and removed based on the number of expressed genes (500–10,000 retained) and mitochondrial proportion (<10% retained). Normalization and variance stabilization used regularized negative binomial regression (sctransform). After initial co-clustering of data from the different preparations, non-neuronal cell clusters were identified by their gene expression profiles. Clusters not expressing high levels of neuronal or somatosensory genes like *SNAP25*, *SCN9A*, *SCN10A*, *PIEZO2*, *NEFH*, etc. but instead expressing elevated levels of markers of non-neuronal cells including *PRP1*, *MBP*, *QKI*, *LPAR1*, and *APOE* were tagged as non-neuronal and were removed to allow reclustering of 'purified' human DRG neurons. A total of 1837 human DRG neuronal nuclei were included in the analysis (Table 1). The mean number of genes detected per nucleus was 2839 (range 501–9652), with a standard deviation of 1917. Doublet detection was performed on the individual datasets using DoubletFinder (McGinnis et al., 2019). For the clustering shown in the main figures the small number of potential doublets (Figure 1—figure supplement 2E) were not removed; principal components (PCs) were determined from integrated assay data and PCs 1–16 were used both for UMAP display of the data and for determining clusters. The resolution for clustering used relatively low stringency (2.0) and closely related clusters without distinguishing markers were merged. The plots shown in the Figures used RNA data that was normalized with the Seurat NormalizeData command and default settings. Raw data deposited in GEO are the

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Nguyen, M. , Ryba, N. and Davidson, S. (2022). Single nuclear capture, sequencing, and data analysis. Bio-protocol Preprint. bio-protocol.org/prep1784.
2. Nguyen, M. Q., von Buchholtz, L. J., Reker, A. N., Ryba, N. J. and Davidson, S.(2021). Single-nucleus transcriptomic analysis of human dorsal root ganglion neurons. eLIFE. DOI: 10.7554/eLife.71752